

Meth d for the screening of $\alpha_2\delta$ -1 subunit binding ligandsFIELD OF THE INVENTION

5 The invention relates to a method for the screening of ligands which bind a soluble secreted cerebral cortical voltage-dependent calcium channel $\alpha_2\delta$ subunit polypeptide, in particular calcium channel $\alpha_2\delta$ -1 subunit.

BACKGROUND OF THE INVENTION

10 Gabapentin (1-aminoethyl-cyclohexane acetic acid) is currently commercialized for the treatment of epilepsy. The compound has however been recognized as being also useful for the treatment of pain and anxiety.

Recent reports have suggested an interaction between gabapentin and the $\alpha_2\delta$ subunit of
15 a voltage-dependent calcium channel (VDCC). But electro-physiological studies have yielded conflicting data on the action of gabapentin at VDCCs, even though the relevance of the interaction of gabapentin at the $\alpha_2\delta$ subunit to the clinical utility of the drug is becoming clearer. However, none of the prototype anticonvulsant drugs displace [3 H]gabapentin binding from the $\alpha_2\delta$ -1 subunit.

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The most frequently used assay currently available for the screening of ligands that bind the $\alpha_2\delta$ subunit involves the use of pig membrane extracts as a source of the $\alpha_2\delta$ subunit. Such an assay presents major inconveniences. Firstly, because the assay material is a membrane extract, it is very difficult to accurately determine the protein composition
25 from one assay preparation to another particularly with regard to the subtype. Also, the presence of various impurities in the assay preparation is a problem in small plate assays. Furthermore, as the protein preparation lacks homogeneity, the interaction between the targeted protein and the assay plate is often quite uneven. This renders the streamlining of the assay in a high throughput format almost impossible to achieve.

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SUMMARY OF THE INVENTION

The inventors have found that it was possible to use a soluble secreted form of a voltage-dependant calcium channel $\alpha_2\delta$ -1 subunit polypeptide (hereinafter $\alpha_2\delta$ -1 subunit polypeptide) in an assay for the screening of ligands which bind the $\alpha_2\delta$ -1 subunit.

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The exact position and configuration of the [³H]gabapentin binding site on the $\alpha_2\delta$ subunit is not currently known. Furthermore, recent deletion experiments on the porcine $\alpha_2\delta$ -1 subunit coding sequence have shown that amino-acids close to the C-terminal region are needed in order for the protein to bind [³H]gabapentin. For this very reason,

10 the use of truncated forms of the porcine $\alpha_2\delta$ -1 subunit in screening assays has not been disclosed or suggested in the prior art because there was concern as to whether relevant levels of binding capacity would be achieved in an assay environment.

15 The assay of the invention is of considerable interest because it confirms that a recombinant soluble secreted $\alpha_2\delta$ -1 subunit polypeptide can be used in high throughput $\alpha_2\delta$ -1 ligand screening. It also provides a useful advantage over the pig membrane extract screening assay as it allows the study of $\alpha_2\delta$ -1 subtype-specific binding ligands. Proteins can be tagged which makes purifying convenient and possible to use a tagged antibody for recognition.

20 It was not clear whether the addition of the 6His tag to the C-terminus of the protein would affect the [³H]gabapentin binding properties of $\alpha_2\delta$.

It was also unclear whether a C-terminally located 6His tag on $\alpha_2\delta$ would be accessible for interaction with the Ni NTA chromatography matrix (for purification purposes) and SPA bead, or Ni flashplate well surface (for purposes of the assay).

25 The invention concerns a method for the screening of ligands which bind a calcium channel $\alpha_2\delta$ -1 subunit.

The method comprises the steps of:

- contacting a secreted soluble recombinant calcium channel $\alpha_2\delta$ -1 subunit polypeptide with:

30 - a ligand of interest; and
- a labelled compound which binds a $\alpha_2\delta$ -1 subunit; and

- measuring the level of binding of the labelled compound to the secreted soluble $\alpha_2\delta$ -1 subunit.

5 The invention also concerns a method for the screening of biologically active products, in particular products that modulate a nervous system function in a subject, comprising the steps of:

- contacting a secreted soluble recombinant calcium channel $\alpha_2\delta$ -1 subunit polypeptide with:

10 - a candidate product; and

- a labelled compound which binds a $\alpha_2\delta$ -1 subunit; and

- measuring the level of binding of the labelled compound to the secreted soluble $\alpha_2\delta$ -1 subunit.

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The invention also concerns a kit for the screening of ligands which bind a calcium channel $\alpha_2\delta$ -1 subunit.

The kit comprises:

- a secreted soluble recombinant calcium channel $\alpha_2\delta$ -1 subunit

20 - polypeptide; and

- a labelled compound which binds a calcium channel $\alpha_2\delta$ -1 subunit.

The invention also concerns a kit or a method for the screening of ligands which bind a calcium channel $\alpha_2\delta$ subunit, a method for the screening of biologically active products,

25 in particular products that modulate a nervous system function in a subject, characterized in that said kits or methods comprise at least one of the following compounds :

1) A calcium channel $\alpha_2\delta$ subunit that is soluble and retain the functional characteristics of the full-length or wild-type $\alpha_2\delta$ subunit from which it derives.

30 2) A calcium channel $\alpha_2\delta$ subunit according to 1) wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives is of mammalian origin.

3) A calcium channel $\alpha_2\delta$ subunit according to 2) wherein the mammalian origin is a human, a porcine, a rat or a mouse origin.

4) A calcium channel $\alpha_2\delta$ subunit according to 3) wherein the mammalian origin is a human origin.

5) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 4) above, wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives is naturally expressed in the cerebral cortical.

6) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 5) above, wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives is voltage-dependent.

10 7) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 6) above, wherein the $\alpha_2\delta$ subunit is cleaved.

8) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 7) above, wherein the $\alpha_2\delta$ subunit is cleaved into separate α_2 and δ peptides.

9) A calcium channel $\alpha_2\delta$ subunit according to 8), wherein the α_2 and δ peptides are disulfide-bridged.

15 10) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 6) above, wherein the $\alpha_2\delta$ subunit is not cleaved.

11) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 10) above characterized in that it is purified or isolated.

20 12) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 11) above characterized in that it is processed as the full-length or wild-type $\alpha_2\delta$ subunit from which it derives is naturally processed.

13) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 12) above characterized in that it is producable by the baculovirus/insect cells expression system.

25 14) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 13) above characterized in that it is produced by the baculovirus/insect cells expression system.

15) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 14) above characterized in that its δ peptide comprises at least the ligand-interacting part(s) of the complete δ peptide from which it originates

30 16) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 15) above characterized in that its δ peptide has a C-terminal truncation with respect to the complete δ peptide from which it originates, said truncation being sufficient to render the truncated δ peptide soluble.

17) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 16) above characterized in that its α_2 peptide comprises at least the ligand-interacting part(s) of the complete α_2 peptide from which it originates.

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18) A calcium channel $\alpha_2\delta$ subunit according to any one of 15) or 17) above characterized in that ligand is gabapentin, L-Norleucine, L-Allo-Isoleucine, L-Methionine, L-Leucine, L-Isoleucine, L-Valine, Spermine or L-Phenylalanine.

19) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 18) above characterized in that its α_2 peptide comprises at least the ligand-interacting part(s) of the complete α_2 peptide from which it originates, its δ peptide comprises at least the ligand-interacting part(s) of the complete δ peptide from which it originates and its δ peptide does not comprise a part of the transmembrane domain of the complete δ peptide from which it originates which renders said calcium channel insoluble.

10 20) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 19) above wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives or originates is $\alpha_2\delta$ -1, $\alpha_2\delta$ -2, $\alpha_2\delta$ -3 or $\alpha_2\delta$ -4.

15 21) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 20) above wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives or originates has the amino acid sequence of SEQ ID N°20.

22) A calcium channel $\alpha_2\delta$ subunit according to 20) or 21) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of SEQ ID N° 4, SEQ ID N° 5 or SEQ ID N° 6.

20 23) A calcium channel $\alpha_2\delta$ subunit according to any one of 20) to 22) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of the region comprised between amino acid number 340 and amino acid number 1062 of SEQ ID N°20.

25 24) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 20) above wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives or originates has the amino acid sequence of SEQ ID N°21.

25 25) A calcium channel $\alpha_2\delta$ subunit according to 20) or 24) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of SEQ ID N° 10, SEQ ID N° 11 or SEQ ID N° 12.

30 26) A calcium channel $\alpha_2\delta$ subunit according to any one of 20), 24) or 25) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of the region comprised between amino acid number 306 and amino acid number 1019 of SEQ ID N°20.

35 27) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 20) above wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives or originates has the amino acid sequence of SEQ ID N°55.

28) A calcium channel $\alpha_2\delta$ subunit according to 20) or 27) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of SEQ ID N° 53, SEQ ID N° 54 or SEQ ID N° 55.

29) A calcium channel $\alpha_2\delta$ subunit according to any one of 20), 27) or 28) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of the region comprised between amino acid number 302 and amino acid number 1050 of SEQ ID N°55.

30) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 20) above wherein the full-length or wild-type $\alpha_2\delta$ subunit from which it derives or originates has the amino acid sequence of SEQ ID N°33 or SEQ ID N°44.

31) A calcium channel $\alpha_2\delta$ subunit according to 20) or 30) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of SEQ ID N° 34, SEQ ID N° 35, SEQ ID N° 36, SEQ ID N° 41, SEQ ID N° 42 or SEQ ID N° 43.

32) A calcium channel $\alpha_2\delta$ subunit according to any one of 20), 30) or 31) above characterized in that the amino acid sequence of its unprocessed form comprises or consists of the region comprised between amino acid number 302 and amino acid number 1018 of SEQ ID N°33 or SEQ ID N°44.

33) A calcium channel $\alpha_2\delta$ subunit according to any one of 20), 30) or 31) above characterized in that the amino acid sequence of its unprocessed form comprises the region comprised between amino acid number 302 and amino acid number 1018 of SEQ ID N°33 or SEQ ID N°44.

34) A calcium channel $\alpha_2\delta$ subunit according to any one of 20), 30), 31), 32) or 33) above characterized in that its α_2 peptide comprises the region comprised between amino acid number 302 and amino acid number 946 or 997 of SEQ ID N°33 or of SEQ ID N°44 and its δ peptide comprises the region comprised between amino acid number 984 and amino acid number 1018 of SEQ ID N°33 or of SEQ ID N°44.

35) A calcium channel $\alpha_2\delta$ subunit characterized in that its α_2 peptide and its δ peptide have 99%, 98%, 97%, 96%, or 95% homology or identity with the α_2 peptide and the δ peptide respectively of a calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 34) above.

36) A nucleic acid molecule characterized in that its nucleotide sequence comprises a nucleotide sequence which encodes a calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 35).

37) A nucleic acid molecule characterized in that its nucleotide sequence comprises a nucleotide sequence which encodes the α_2 peptide or the δ peptide of a calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 35).

38) A nucleic acid molecule which hybridizes under stringent conditions with a nucleic acid molecule according to 36) or 37) above or 39) herebelow.

39) A nucleic acid molecule according to any one of 36) to 38) above which comprises SEQ ID N°1, SEQ ID N°2, SEQ ID N°3, SEQ ID N°7, SEQ ID N°8, SEQ ID N°9, SEQ ID N°13, SEQ ID N°14, SEQ ID N°15, SEQ ID N°30, SEQ ID N°31, SEQ ID N°32, SEQ ID N°38, SEQ ID N°39, SEQ ID N°40, SEQ ID N°50, SEQ ID N°51, or SEQ ID N°52.

5 40) A vector capable of expressing a nucleic acid molecule according to any one of 36) to 39) above.

10 41) An expression vector comprising a nucleic acid molecule according to any one of 36) to 39) above.

42) A vector according to 40) or 41) above which is a baculovirus vector.

43) A cell comprising a nucleic acid molecule according to any one of 36) to 39) above.

44) A cell comprising a vector according to 40), 41) or 42) above.

15 45) A cell according to 43) or 44) above which is a mammalian cell or an insect cell.

46) A composition comprising a calcium channel $\alpha_2\delta$ subunit according to any one of 7) to 9) above and a calcium channel $\alpha_2\delta$ subunit according to 10) above.

47) A calcium channel $\alpha_2\delta$ subunit according to any one of 1) to 35) above, characterized in that it is tagged or labelled.

20 48) A nucleic acid molecule according to any one of 36) to 39) above, characterized in that it is tagged or labelled.

49) A calcium channel $\alpha_2\delta$ subunit according to 47), characterized in that it is tagged at the C-terminal part of the δ peptide.

50) A nucleic acid molecule according to 48) above, characterized in that it is tagged at

25 the end of the region of the nucleic acid molecule encoding the C-terminal part of the δ peptide.

51) A calcium channel $\alpha_2\delta$ subunit according to 47) or 49), characterized in that it is tagged by a 6 histidine residue tagg.

52) A nucleic acid molecule according to 48) or 50), characterized in that it is tagged by a

30 6 histidine residue tagg.

The invention also concerns a screening assay using a compound according to any one of 1) to 52) above. This screening assay is preferably an SPA assay, a Flashplate assay, a Nickel Flasplate assay, a Filter binding assay or a Wheat Germ Lectin flasplate assay.

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The invention also concerns the use of screening assay using a compound according to any one of 1) to 52) above to detect or measure the binding or interaction of a ligand of a

calcium channel $\alpha_2\delta$ subunit and a calcium channel $\alpha_2\delta$ subunit. Specific ligands of interest are gabapentin, L-Norleucine, L-Allo-Isoleucine, L-Methionine, L-Leucine, L-Isoleucine, L-Valine, Spermine or L-Phenylalanine.

5 The above screening assays or uses are preferably an SPA assay, a Flashplate assay, a Nickel Flasplate assay, a Filter binding assay or a Wheat Germ Lectin flasplate assay.

The invention also concerns a kit to detect or measure the binding or interaction of a ligand of a calcium channel $\alpha_2\delta$ subunit and a calcium channel $\alpha_2\delta$ subunit characterized 10 in that it comprises a compound according to any one of 1) to 52). Specific ligands of interest are gabapentin, L-Norleucine, L-Allo-Isoleucine, L-Methionine, L-Leucine, L-Isoleucine, L-Valine, Spermine or L-Phenylalanine.

15 The kits of the invention recited above are usable in an SPA assay, a Flashplate assay, a Nickel Flasplate assay, a Filter binding assay or a Wheat Germ Lectin flasplate assay.

There is a great advantage to use imidazole in the context of the assays and kits of the invention because it increases the possibilities of interactions between the ligand and the receptor. A preferred range of use of imidazole in an SPA assay of the invention is 20 between about 1 mM and about 50 mM. A preferred range of use of imidazole in a Flasplate assay of the invention is between about 1 mM and about 20 mM.

25 There is a great advantage to use Bovine Serum Albumine (BSA) in the context of the assays and kits of the invention because it renders the compounds used in these assays and kits a lot more stable and therefore allows their in large-scale screening, especially in an high-throughput screening format. A preferred range of use of BSA is between about 0.025% and about 0.05%.

30 A great advantage of the compounds described above which are used or comprised in the assays and kits of the invention is that they can be obtained and therefore used as an homogeneous material.

35 Another great advantage of the compounds described above and used or comprised in the assays and kits of the invention is that they can be obtained in large quantity by the technique of the DNA recombinant. A most preferred system to produce these compounds is the baculovirus/insect cells system which furthermore allows to obtain high yields of production.

Precise embodiments of the above kits and assays are recited in the continuation of the text of this application, especially in the examples.

The invention also resides in a product or ligand isolated, identified or selected using the 5 above screening methods or kits, for use as a medicament or as a lead for further drug development purposes. As indicated above, the compounds are potentially useful for treating disorders of the nervous system, including epilepsy, pain and anxiety

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BRIEF DESCRIPTION OF THE FIGURES

Figure 1 represents the elution profile of the recombinant polypeptide with the amino acid sequence of SEQ ID No 37 purified by Superdex-200 chromatography, either before or after electron on NI-NTA.

15 Figure 2 illustrates the optimization of imidazole concentrations in an embodiment of the SPA assay of the invention. SPA assay of [³H]gabapentin (18.4nM) binding to the recombinant polypeptide of amino acid sequence SEQ ID No 9 (s- $\alpha_2\delta$ -1b-6His) (20 μ l).

20 Figure 3 illustrates the optimization of imidazole concentrations in an embodiment of the flashplate assay of the invention. Flashplate assay of [³H]gabapentin (14nM) binding to the recombinant polypeptide of amino acid sequence SEQ ID No 37 (s- $\alpha_2\delta$ -1b-6His) (10 μ l).

25 Figure 4 illustrates the flashplate time course of [³H]gabapentin (13 nM) binding to various concentrations of the recombinant polypeptide with the amino acid sequence of SEQ ID No 37.

30 Figure 5 illustrates the capacity of the recombinant polypeptide with the amino acid sequence of SEQ ID No 37 in a flashplate assay after 3 hours of incubation.

Figure 6 illustrates the optimum imidazole concentration, assayed after 3 hours of incubation, required to maximize [³H]gabapentin binding using a constant amount of the recombinant polypeptide with the amino acid sequence of SEQ ID No 37.

Figure 7 illustrates flashplate assay of [³H]gabapentin saturation binding to the purified recombinant polypeptide with the amino acid sequence of SEQ ID No 37, assayed after 3 hours of incubation.

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Figure 8 illustrates the flashplate time course optimisation of imidazole concentration required to maximize the [³H]Leucine (10.1 nM) binding window to the purified recombinant polypeptide with the amino acid sequence of SEQ ID No 37, assayed after 3 hours of incubation.

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Figure 9 illustrates competition curves of three compounds in the flashplate assay format, assayed after 3 hours of incubation (see also Table 2).

Figure 10 illustrates the dose response nature of [³H]gabapentin binding to s- $\alpha_2\delta$ -1b-6His 15 in the Wheat Germ lectin flashplate assay. The level of non-specific binding is low (around 50-70cpm) and stable, independent of the volume of protein assayed or the point analysed on the time-course. The window is relatively stable over an extended period of time with just a gradual decline from the 15-hour time point (approximately 10% of the window every 24 hours).

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Figure 11 illustrates the dose response nature of [³H]gabapentin binding to $\alpha_2\delta$ -1 and the maintenance of a constant low-level of non-specific binding (around 30-60cpm) independent of protein volume assayed.

25 Figure 12 illustrates the dose response nature of [³H]gabapentin binding to $\alpha_2\delta$ -1 in the Nickel flashplate assay. The level of non-specific binding is low (around 70-100cpm) and stable, independent of the volume of protein assayed or the point analysed on the time-course. A stable window is maintained for a period of at least 50 hours (between ~20 and 70 hours on the time-course).

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Figure 13 illustrates the stability of $\alpha_2\delta$ -1/[³H] gabapentin binding assay. Each well was performed in triplicate and contains in 200 μ l of : 0.1M HEPES pH7.3, 0.5mM Imidazole Various concentrations of BSA fraction V (0, 0.025, 0.05, 0.25, 0.5 and 1%).

Figure 14 is an example of amino acid sequence alignments of the $\alpha_2\delta$ -1 deletion region in four species variants of $\alpha_2\delta$ -1 splice isoform b.

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DETAILED DESCRIPTION OF THE INVENTION

The invention concerns a method for the screening of ligands which bind a soluble secreted $\alpha_2\delta$ subunit polypeptide, in particular a soluble secreted $\alpha_2\delta$ -1 subunit polypeptide. The term $\alpha_2\delta$ subunit polypeptide, when used herein, is intended to designate a structure containing two polypeptides (α_2 and δ) attached or associated to one another, in particular by covalent disulfide bridges. More particularly, the targeted $\alpha_2\delta$ subunit binding site is preferably the [3 H]gabapentin binding site. The various parameters of the method of the invention are described in further detail below.

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A – Secreted soluble recombinant $\alpha_2\delta$ -1 subunit polypeptide

Several nucleotide sequences encoding a secreted soluble form of an $\alpha_2\delta$ -1 subunit can be used in the context of the present invention. Preferred soluble secreted $\alpha_2\delta$ -1 subunit polypeptides are derived from eukaryotic $\alpha_2\delta$ -1 subunits, more preferably from mammal, 20 such as mouse, rat, rabbit, porcine, bovine or others and human $\alpha_2\delta$ -1 subunits. Most preferred soluble secreted $\alpha_2\delta$ -1 subunit polypeptides are derived from the human or porcine $\alpha_2\delta$ -1 subunits.

More specifically, the selected nucleotide sequences encode a secreted soluble 25 polypeptide having at least 80%, preferably 90%, more preferably 95%, and most preferably 98 or 99% amino-acid identity with the polypeptide comprising from amino acid 1 to between amino-acids 985 and 1087 of SEQ ID N°33 or SEQ ID N°44, preferably between amino-acids 985 and 1085, more preferably between amino-acids 985 and 1078, most preferably between amino-acids 985 and 1064, 1059 or 1044 of SEQ ID 30 N°33 or SEQ ID N°44.

In particular embodiments, selected nucleotide sequences encode a secreted soluble polypeptide having at least 80%, preferably 90%, more preferably 95%, and most preferably 98 or 99% amino-acid identity with the polypeptide comprising from amino acid 1 to between amino-acids 1008 and 1087 of SEQ ID N°33 or SEQ ID N°44, in 5 particular between 1018 and 1078, specifically between 1043 and 1078.

More specifically, the selected nucleotide sequences encode a secreted soluble polypeptide having at least 80%, preferably 90%, more preferably 95%, and most preferably 98 or 99% amino-acid identity with the polypeptide comprising from amino 10 acid 1 to between amino-acids 985 and 1054, preferably between amino-acids 985 and 1059, and most preferably between amino-acids 1019 and 1044 of SEQ ID N°33 or SEQ ID N°44.

In order to determine the optimal deletions on the $\alpha_2\delta$ -1 subunit cDNA that yield a 15 soluble secreted polypeptide devoid of membrane anchorage structures and having a functional [³H]gabapentin binding site, the inventors tested the expression of several human or porcine $\alpha_2\delta$ -1 subunit cDNA deletion mutants. The discussion provided below provides detailed comments on possible truncations, giving as an example the porcine $\alpha_2\delta$ -1 subunit. However, given the very substantial cross-species homology for $\alpha_2\delta$ -1 20 subunit sequences, the comments below can also be applied to other eukaryotic species, and more particularly other mammalian species such as the rat, the mouse or the rabbit. Their $\alpha_2\delta$ -1 subunit sequences, which are available in public databases, share a very substantial homology with the human and porcine $\alpha_2\delta$ -1 subunit sequences. Therefore, the inventore believe that this succesfull identification and characterization of a region 25 in $\alpha_2\delta$ -1 which can be modified to produce a soluble $\alpha_2\delta$ -1 of pig and human whilst maintaining Gabapentin binding, enables those skilled in the art to modify other species $\alpha_2\delta$ -1 polypeptides. Exambles of this modification site in other $\alpha_2\delta$ -1 from other species is shown in figure 14.

The inventors found that by deleting from the porcine $\alpha_2\delta$ -1 subunit cDNA a nucleotide 30 sequence encoding as much as amino-acids 967 to 1091 of the native protein, soluble polypeptides could be obtained. On the other hand, the minimal deletion required to achieve solubility appears to be located around nucleotides encoding amino-acids 1064

to 1091 of the sequence of SEQ ID N°33. In this regard, the mutant polypeptide expressed using a cDNA deletion mutant from which a sequence encoding amino-acids 1064 to 1091 is removed is found in both soluble and membrane-associated forms, with $[^3\text{H}]$ gabapentin and/or other derivatives or compounds such as pregabalin and 5 gabapentoids binding properties similar to that of the wild type protein. Furthermore, a mutant protein expressed using a cDNA deletion mutant from which a nucleotide sequence encoding amino-acids 1085 to 1091 is removed recovers its membrane anchorage properties. Also, mutant proteins expressed using cDNA deletion mutants from which nucleotide sequences encoding either amino-acids 1037 to 1091 or amino-10 acids 1019 to 1091 of SEQ ID N°33 or 44 are removed are found in soluble form.

The inventors believe that the soluble secreted $\alpha_2\delta$ -1 subunit polypeptides which are as close as possible to the native sequence and which are therefore more likely to retain their native folding and hence their $[^3\text{H}]$ gabapentin- binding properties are those 15 corresponding to a protein in which amino-acid stretch 985-1091 to 1074-1091, the amino-acid sequence of SEQ ID N°33 or 44 has been deleted. The skilled scientist can quite easily determine within this amino-acid stretch the optimal mutant protein.

The invention therefore particularly concerns a screening assay in which the secreted 20 soluble $\alpha_2\delta$ -1 subunit polypeptide is preferably a polypeptide having at least 80% identity with the polypeptide comprising from amino-acid 1 to between amino-acid 985 and 1054, preferably between amino-acids 985 and 1059, and most preferably between amino-acids 1019 and 1064 of SEQ ID N°33 or SEQ ID N°44. Preferred $\alpha_2\delta$ -1 subunit 25 polypeptides which can be used in the present invention are those of SEQ ID N°34, 35, 36, 37, 41, 42 and 43, with the polypeptides of SEQ ID N°33 and SEQ ID N°43 being most preferred.

In a first and preferred embodiment of the invention, the $\alpha_2\delta$ -1 subunit polypeptide is purified before it is used in the assay. The purification step, an example of which is provided further in this specification, can be carried out using several purification 30 techniques well-known to the skilled person.

In some instances, it is required to tag the $\alpha_2\delta$ -1 subunit polypeptide prior to purification. The tag is then in most instances encoded into the nucleotide sequence that is needed to

express the polypeptide. Examples of such tags include, but are not limited to sequences encoding C-myc, FLAG, a sequence of histidine residues, heamaglutin A, V5, Xpress or GST. Most of these tags can be incorporated directly into the sequence, for instance through PCR amplification by incorporating the appropriate coding sequence in one of 5 the PCR amplification primers. However, the tag can also be introduced by other means such as covalent binding of the appropriate nucleic acid sequence encoding the tag moiety with the 5' or 3' end of the nucleic acid sequence encoding the polypeptide sequence. This is the case for GST. It should be noted that the tag can be located at either 10 end of the polypeptide sequence. Furthermore, in some instances, it can be advantageous to insert a cleavage site between the tag and the polypeptide sequence in order to permit removal of the tag sequence if needed. Therefore a first object of this invention of the invention is the use of C-myc, FLAG, a sequence of histidine residues, heamaglutin A, V5, Xpress or GST taggs for the purification and screening of $\alpha_2\delta$ -1. Preferably, the use 15 of C-myc, FLAG, a sequence of histidine residues, heamaglutin A, V5, Xpress or GST taggs for the purification and screening of $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38. More preferably, the use of a sequence of histidine residues tagg for the purification and screening of $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38, more preferably, the use of a sequence of 6 histidine residues tagg for the purification and screening of $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38 and most 20 preferably the use of C-terminal a 6 histidine residues tagg of $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38 for the purification and screening

A secont object of the invention is the use of nucleic acids encoding C-myc, FLAG, a sequence of histidine residues, heamaglutin A, V5, Xpress or GST taggs for the purification and screening of expressed $\alpha_2\delta$ -1. Preferably, the use of nucleic acids 25 encoding C-myc, FLAG, a sequence of histidine residues, heamaglutin A, V5, Xpress or GST taggs for the purification and screening of expressed $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38. More preferably, the use of nucleic acids encoding a sequence of histidine residues tagg for the purification and screening of an expressed $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38, more preferably, the use nucleic acid sewuence 30 encoding a sequence of 6 histidine residue tagg for the purification and screening of an expressed $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38 and most preferably the use of C-terminal a nucleic acid sequence encoding 6 histidine residue tagg of

expressed $\alpha_2\delta$ -1 of SEQ ID N°30, 31, 32, 33, 34, 35, 36, 37 or 38 for purification and screening

In other cases, providing a tag to the polypeptide is not needed. For instance, the protein
5 can be purified using affinity columns loaded with specific monoclonal antibodies.

In a second embodiment of the invention, the $\alpha_2\delta$ -1 subunit polypeptide can be only partially purified. For instance, it can be purified along with other contaminating proteins using an appropriate chromatography matrix such as ion-exchange chromatography
10 column. In such instances, it is not required to tag the desired polypeptide of interest.

The most preferred embodiment contemplated by the inventors concerns the use of a purified tagged $\alpha_2\delta$ -1 subunit polypeptide. A particularly preferred tag is a nucleotide sequence encoding from 2 to 10, and preferably 6 histidine residues as provided in the
15 polypeptide of SEQ ID No 37.

The polypeptide may be prepared by expression of the corresponding nucleic acid molecule in any appropriate host cell, using various vector systems known to the skilled person. Typical examples of suitable cells include prokaryotic and eucaryotic cells, such
20 as bacteria, yeasts, mammalian cells (including human cells), insect cells, etc. The vector may be a plasmid, virus, episome, phage, etc. As illustrated in the examples, the polypeptide can be produced for instance by expression of the nucleic acid in insect cells, using a baculovirus vector.

25 A most preferred system of expression of the calcium channel $\alpha_2\delta$ of the invention is the baculovirus/insect cell system. In fact, this system of expression allows to produce only the soluble form, is easy to use because the insect cells can be cultured without adherency and results in very high yield of production. Thus, this system allows mass-production of the calcium channel $\alpha_2\delta$ of the invention, provides an homogeneous
30 production and is therefore particularly suitable for the preparation of this target for screening, in particular for high-throughput screening.

With regard to the $\alpha_2\delta$ -1 subunit polypeptide used subsequently in the screening assay of the invention, several possibilities are also open to the skilled person.

5 In a first and preferred embodiment, the $\alpha_2\delta$ -1 subunit polypeptide comprises a tag moiety which can be selected among the tags referred to above. Such tagged polypeptides are particularly useful in SPA or flashplate assays. A preferred tag is the nucleotide sequence encoding histidine residues referred to above.

10 In a second embodiment, the $\alpha_2\delta$ -1 subunit polypeptide can be used without a tag. This is the case for instance in SPA or flashplate assays comprising beads or plates coated with wheat germ lectin. In such an embodiment, the tag is not needed as the carbohydrate moieties of the $\alpha_2\delta$ -1 subunit polypeptide bind directly to the wheat germ lectin-coated beads or plates.

15 **B - Labelled compounds which bind the $\alpha_2\delta$ -1 subunit polypeptide**

In cases where the $\alpha_2\delta$ -1 binding site is the [³H]gabapentin binding site, the preferred labelled compound which can be used is of course gabapentin itself. However, gabapentin is not the only labelled compound which can be used in this context. Indeed, it has been previously demonstrated that saturation binding analyses on porcine synaptic 20 plasma cerebral cortex membranes performed in the presence of L-leucine indicate a competitive interaction of the amino acid with the [³H]gabapentin binding site, significantly reducing [³H]gabapentin binding affinity for the site. The inventors believe that this competitive interaction is true across all the amino-acids listed in table 1 below.

25 **Table 1**

Binding affinities of selected amino acids ($IC_{50} < 500nM$) for the [³H]gabapentin site in porcine cortical membranes

	<u>COMPOUND</u>	<u>IC_{50} (nM) ARITHMETIC MEAN (N=3) \pm S.E.M.</u>
30	Gabapentin	42.1 ± 5.5
	L-Norleucine	23.6 ± 6.7
	L-Allo-Isoleucine	32.8 ± 6.0

L-Methionine	49.6 ± 10.0
L-Leucine	61.3 ± 20.9
L-Isoleucine	68.8 ± 1.9
L-Valine	330 ± 18
5 L-Phenylalanine	351 ± 89

It is therefore possible to use commercially available labelled forms of these high affinity ligands in replacement of gabapentin. The utility of [³H]L-leucine has been demonstrated in a filter binding assay and in a flashplate assay format. The inventors believe that 10 labelled amino acids but also other compounds, with affinities preferably below 500 nM in the binding assay can be used as replacements of gabapentin.

With regard to the label, several embodiments can be used in the context of the invention. Preferred labels are of course radioactive labels, a list of which is provided 15 further in this specification.

An object of this invention is the use of labelled compounds which have an affinity of less than 500nm for the gabapentin binding site of $\alpha 2\delta-1$ for the screening of ligands that bind to $\alpha 2\delta-1$, preferably the use of labelled Gabapentin, L-Norleucine, L-Allo-Isoleucine, L-Methionine, L-Leucine, L-Isoleucine, L-Valine or L-Phenylalanine, more 20 preferably the use of labelled Gabapentin.

C - Assay formats and conditions

Several assay formats can be used to carry out the method of the present invention. 25 Preferred assay formats include scintillation assays such as the scintillation proximity assay (SPA) or the flashplate assay. Other assay formats well known to those skilled in the arts such as the filter binding assay and the centrifugation assay are also contemplated in the present invention.

30 SPA and flashplate assays are preferred assay formats for the present invention. Additional details on these assays are provided below.

Scintillation assay format

Scintillation assays technology either involves the use of scintillant beads (for the SPA assay) or plates (for the flashplate assay). SPA beads are usually made from either cerium-doped yttrium ion silicate ($y_2SiO_5:Ce$) or polyvinyltoluene (PVT) containing an 5 organic scintillant such as PPO. Flashplates commonly used are those such as Ni chelate flashplates although other flashplates can also be used.

Assays are usually carried out in aqueous buffers using radioisotopes such as 3H , ^{125}I , ^{14}C , ^{35}S or ^{33}P that emit low-energy radiation, the energy of which is easily dissipated in 10 an aqueous environment. For example, the electrons emitted by 3H have an average energy of only 6 keV and have a very short path length (-1 ~tm) in water. If a molecule labelled with one of these isotopes is bound to the bead or flashplate surface, either directly or via interaction with another molecule previously coupled to the bead or flashplate, the emitted radiation will activate the scintillant and produce light. The 15 amount of light produced, which is proportional to the amount of labelled molecules bound to the beads, can be measured conveniently with a liquid scintillation (LS) counter. If the labelled molecule is not attached to the bead or a flashplate surface, its radiation energy is absorbed by the surrounding aqueous solvent before it reaches the bead, and no light is produced. Thus, bound ligands give a scintillation signal, but free 20 ligands do not, and the need for a time- consuming separation step, characteristic of conventional radioligand binding assays, is eliminated. The manipulations required in the assays are reduced to a few simple pipetting steps leading to better precision and reproducibility..

The conditions under which SPA and flashplate assays are performed in the context of 25 the present invention are provided below.

Scintillation assay conditions**1) SPA assay**

The SPA assays is first developed to optimize the conditions under which the radioligand 30 binds the $\alpha_2\delta-1$ subunit polypeptide. The parameters which can be varied to optimize radioligand binding in a typical SPA assay using Amersham beads include assay temperature, $\alpha_2\delta-1$ subunit polypeptide interaction with the radioligand and the SPA beads, radioligand concentration as well as pH variations.

The temperature at which the assay can be carried out can vary from 1 to 30°C. Preferred temperatures range from 18 to 23°C, with 21°C being the most preferred temperature. The interaction of the $\alpha_2\delta$ -1 subunit polypeptide with the SPA beads can be

5 optimized by adjusting the concentration of the polypeptide and by introducing a reagent which will favor this interaction. When 50 mg of Amersham SPA beads are used, the $\alpha_2\delta$ -1 subunit polypeptide concentration may vary from 0.1 to 10 pmoles per well, with the optimal concentration being generally around 5 to 6 pmoles per well.

10 As for the reagent favoring the interaction between the $\alpha_2\delta$ -1 subunit polypeptide and the radioligand as well as the Amersham SPA beads, the inventors found that imidazole could be efficiently used for that purpose when the $\alpha_2\delta$ -1 subunit polypeptide is tagged with an amino acid sequence including 6 histidine residues. Furthermore, and more importantly, it was found that imidazole also enhanced binding of the radioligand to the

15 $\alpha_2\delta$ -1 polypeptide.

The optimal concentration of imidazole used to enhance radioligand binding varies depending on the concentration of $\alpha_2\delta$ -1 subunit polypeptide used in the assay. For instance, when the volume of the $\alpha_2\delta$ -1 subunit polypeptide is about 20 μ l ($\alpha_2\delta$ -1

20 polypeptide concentration of 0.6 pmol/ μ l), imidazole concentrations ranging from 10 to 50 mM can be used, with concentrations ranging between 10 and 30 mM being preferred. A most preferred imidazole concentration is 20 mM. It is to be noted that other compounds such as histidine can be used to enhance radioligand binding. Furthermore, pH variations can also influence radioligand binding although pH variations should be

25 closely monitored as they may have an effect on the structural configuration of the $\alpha_2\delta$ -1 subunit polypeptide. Although the use of imidazole is preferred to enhance radioligand binding, the person skilled in the art knows that the use of imidazole is preferred but is absolutely not essential.

30 The concentration of the radioligand is evaluated with respect to the concentration of $\alpha_2\delta$ -1 subunit polypeptide present in the assay medium. Generally, the concentration of radioligand varies from 1 nM to 100 nM. A preferred [3 H]gabapentin concentration is

about 5 to 20 nM, with a most preferred concentration being about 10 nM. A preferred [³H]leucine concentration is also about 5 to 20 nM, with a most preferred concentration being about 10 nM. It is to be noted that the concentration of other radioligands having affinities similar to those of [³H]gabapentin and [³H]leucine should also be in the range 5 of about 5 to 20 nM.

Once the optimal radioligand binding conditions have been determined, a test ligand can be introduced in the assay medium to evaluate the level of displacement of the radioligand. The concentration of test ligand to be introduced in the assay medium usually varies from 0.1 nM to about 100 μ M. A preferred test ligand concentration of 10 about 10 μ M is usually a starting point in a high throughput screening assay. Then, depending on the number of hits obtained, it may be lowered or increased.

It is to be noted that the parameters set forth above, which have been evaluated for a typical SPA assay using Amersham SPA beads can be adjusted by the skilled person, for 15 example if SPA beads of a different type are used.

2) Flashplate assay

Similarly to the SPA assays, the flashplate can first be developed in order to optimize the conditions under which the radioligand binds the $\alpha_2\delta$ -1 subunit polypeptide. The 20 parameters which can be varied to optimize radioligand binding in a typical flashplate assay using NEN Ni chelate flashplates or Wheat Germ lectin flashplates also include assay temperature, $\alpha_2\delta$ -1 subunit polypeptide interaction with both the radioligand and the flashplates, radioligand concentration as well as pH variations.

25 The temperature at which the assay can be carried out can vary from 1 to 30°C. Preferred temperatures range from 18 to 23°C, with 21°C being the most preferred temperature.

30 The interaction of the $\alpha_2\delta$ -1 subunit polypeptide with the flashplates can be optimized by adjusting the concentration of the polypeptide and by introducing a reagent which will favor this interaction. When a standard NEN Ni chelate flashplate is used, the $\alpha_2\delta$ -1 subunit polypeptide volume usually varies between 0.5 and 20 μ l for a concentration of

$\alpha_2\delta$ -1 subunit polypeptide of 0.6 pmol/ μ l. As the published maximum binding capacity of NEN plates is about 6 pmol per well, the inventors consider that an optimal concentration of $\alpha_2\delta$ -1 subunit polypeptide is probably around 5 pmol per well at 8 μ l.

5 Although the use of imidazole is preferred to enhance radioligand binding, the person skilled in the art knows that the use of imidazole is preferred but is absolutely not essential.

With regard to the reagent favoring the interaction between the $\alpha_2\delta$ -1 subunit polypeptide and the radioligand as well as the flashplates, the inventors found that imidazole could 10 also be efficiently used for that purpose when the $\alpha_2\delta$ -1 subunit polypeptide is tagged with an amino acid sequence including 6 histidine residues. It was also found that imidazole concentrations substantially enhanced binding of the radioligand to the $\alpha_2\delta$ -1 polypeptide. The optimal concentration of imidazole used to enhance radioligand binding varies depending on the concentration of $\alpha_2\delta$ -1 subunit polypeptide used in the assay. For 15 instance, when the volume of the $\alpha_2\delta$ -1 subunit polypeptide is about 10 μ l ($\alpha_2\delta$ -1 polypeptide concentration of 0.6 pmol/ μ l), the optimal imidazole concentration can vary between 1 and 20 mM, with a concentration of about 10 mM being preferred. As mentioned previously, other compounds such as histidine as well as pH variations may be used to enhance radioligand binding.

20

The concentration of the radioligand is evaluated with respect to the concentration of $\alpha_2\delta$ -1 subunit polypeptide present in the assay medium. Generally, the concentration of radioligand varies from 1 nM to 100 nM. A preferred [³H]gabapentin concentration is about 5 to 20 nM, with a most preferred concentration being about 10 nM. A preferred 25 [³H]leucine concentration is also about 5 to 20 nM, with a most preferred concentration being about 10 nM. It is to be noted that the concentration of other radioligands having affinities similar to those of [³H]gabapentin and [³H]leucine should also be in the range of about 5 to 20 nM.

30 Once the optimal radioligand binding conditions have been determined, a test ligand can be introduced in the assay medium to evaluate the level of displacement of the radioligand. The concentration of test ligand to be introduced in the assay medium

usually varies from 0.1 nM to about 100 μ M. A preferred test ligand concentration of about 10 μ M is usually a starting point in a high throughput screening assay. Then, depending on the number of hits obtained, it may be lowered or increased.

The inventors have tested the displacement of a particular radioligand, [3 H]gabapentin, with (S+)-3-isobutyl gaba, (R)-3-isobutyl gaba and gabapentin. The data provided in the examples which follow clearly shows that the assay can be used in high throughput competition studies.

Example 1

10 **Construction of a nucleotide sequence encoding the putative soluble porcine $\alpha_2\delta$ -1b deletion mutant of SEQ ID N°37**

a) Primer design

PCR primers were designed to generate the soluble porcine $\alpha_2\delta$ -1b deletion mutant of SEQ ID N° 37 as follows:

15 5' PCR primer: This was designed to engineer in a KOZAK translation initiation consensus sequence prior to the coding sequence (Kozak *JBC* 266 19867-19870)

3' PCR primer: This was designed to engineer in six histidine residues followed by a stop-codon at the desired location in the coding sequence. In addition to the stop codon the $\alpha_2\delta$ -1 primers also included an *Eco* RI restriction site.

20

The bold region in each primer sequence denotes the 'tagged' region; addition of sequences not present in the template. Primers were custom synthesized by Perkin Elmer Applied Biosystems UK to the ABI ready pure grade, supplied lyophilized then resuspended to 15 μ M in 10mM TE. JB189 and 195 were provided without 5' phosphate

25 groups:

5' primer JB189 (5'-TCGCCACCATGGCTGCTGGCTGCCTGCTG-3', SEQ ID N°20)

3' primer JB195 (5'-

TCGGAATTCCCTCAGTGATGGTGATGGTGATGAGAACACCACAGTCG

30 GT-3', SEQ ID N°21)

Could you please check the bolded regions?

b) PCR protocols for the generation of the $\alpha_2\delta$ -1 deletion mutant**1) Generation of the pcDNA3-porcine- $\alpha_2\delta$ -(+) PCR template**

An oligo dT-primed λ gt10 porcine cerebral cortical cDNA library was screened by ECL

5 (Amersham) using a 2,381-bp *HindIII* fragment (coding sequence 268-2649) of the rabbit skeletal muscle $\alpha_2\delta$ clone (pcDNA3-Rab- $\alpha_2\delta$ -(+)) (supplied by Neurex) as the probe.

A positive insert was identified and subcloned into pBluescript-SK-(+) to generate pB-PC- $\alpha_2\delta$ -1.1. The clone was sequenced on both strands, except for a 711-bp stretch at one end of the clone, which had a high degree of homology to mitochondrial C oxidase.

10 The $\alpha_2\delta$ coding region was homologous to the 3' region of the human neuronal $\alpha_2\delta$ sequence but lacked 926 bp of 5' coding sequence. The missing sequence was obtained by 5'-RACE using total RNA prepared from porcine cerebral cortex. RACE was performed across a *Bgl* I site unique in known $\alpha_2\delta$ sequences (rabbit (accession no. M21948)), rat (accession number M86621), human (accession no. M76559)

15 The sequence derived from the 5' RACE product was used to design a primer (JB042, 5'-GGGGATTGATCTTCGATCGCG-3'; SEQ ID N°18) specific for the 5'-untranslated end of the cDNA. PCR was then performed with *Pfu* DNA polymerase using JB042 and a primer downstream of the *Bgl* I site (JB040, CTGAGATTGGGGTTCTTG, SEQ ID N°19).

20 The PCR product was ligated to Eco RI linkers (5'-GGAATTCC-3') and then digested with Eco RI and *Bgl* I. The 1,564-bp fragment (5' portion of the $\alpha_2\delta$ cDNA) was gel-purified.

Similarly, a 2,303-bp fragment (3' portion of the $\alpha_2\delta$ cDNA) was isolated after digestion

25 of pB-PC- $\alpha_2\delta$ -1.1 with *Bgl* I and Eco RI. The two fragments of $\alpha_2\delta$ cDNA were then ligated to EcoRI-digested pcDNA3 in a three-way ligation. A clone was picked with the full-length $\alpha_2\delta$ sequence in the positive orientation with respect to the cytomegalovirus promoter (pcDNA3-PC- $\alpha_2\delta$ -(+)).

30 **2) PCR protocol**

The following reagents were added to obtain two cocktails labelled 'lower' and 'upper' buffers.

	<i>Lower</i>	μl
	10x <i>Pfu</i> DNA polymerase buffer	25
	10mM dNTP's	5
	100ng/ μl pcDNA3-porcine- $\alpha_2\delta$ -(+)	10
5	15 μM JB189	8.5
	15 μM JB195	8.5
	H ₂ O	193
	<i>Upper</i>	μl
10	10x <i>Pfu</i> DNA polymerase buffer	25
	H ₂ O	220
	2.5units/ μl <i>Pfu</i> DNA polymerase	5

50 μl aliquots of lower buffer were added to each of four 0.5ml eppendorf tubes. To each
15 was added one PCRgem 100 ampliwx bead (PE biosystems). Tubes were heated to 80°C
for 2 minutes then cooled to 4°C. 50 μl of upper buffer was then added to each tube.
Tubes were then cycled on a Stratagene Robo-Cycler according to the following
conditions: 98°C / 1min 30sec, followed by: for 20 cycles 98°C / 45sec, 54°C / 2min,
72°C / 6min, followed by: 72°C / 20min, followed by: hold at 4°C.

20

The 3228bp PCR product was then purified on a QIAquick PCR purification column
(Qiagen) and eluted with 61 μl of H₂O. The following reagents were added to the eluted
DNA: 0.7 μl 10mM ATP, 7 μl 10x Polynucleotide Kinase buffer, 1 μl 1unit/ μl
Polynucleotide Kinase.

25

The above 5' phosphorylation reaction was incubated at 37°C for 1 hour. The reaction
was stopped by incubation at 65°C for 10min. The 3228bp 5' phosphorylated PCR
product was then gel purified from a 1% agarose gel using QIAEX (Qiagen) beads and
eluted in ~50 μl .

30

Example 2**Cloning of the PCR fragments of Example 1 into the Baculovirus transfer vector****pFastBac1**

The PCR products of Example 1 (3228bp JB189/JB195 derived PCR product coding for 5 6His tagged porcine $\alpha_2\delta$ -1b: SEQ ID No 9) were cloned into *Stu* I digested, calf intestinal phosphatase dephosphorylated, phenol chloroform extracted and QIAEX gel purified pFastBac1 (Life Technologies) using the Rapid DNA ligation kit (Roche Diagnostics) transforming XL1-blue ($\alpha_2\delta$ -1b) *E. Coli* cells:

10 **a) Screening for positive recombinants**

Given that the PCR product was cloned by blunt-end ligation a screen was required to select a recombinant with the gene ligated in the positive orientation with respect to the polyhedrin promoter in pFastBac1. This was achieved by restriction digest of miniprep DNA (Qiagen miniprep kit) prepared from colony minicultures and analysis on a 1% 15 TAE agarose gel. A positive clone was identified according to the following digest patterns:

SEQ ID No 9 in pFastBac1

Eco RI digest performed on miniprep DNA

	Predicted fragments (bp)
PCR product cloned in a positive orientation	4773 and 3230
PCR product cloned in a negative orientation	7989 and 14

b) Sequencing analysis of selected clones

25 One positive was selected for this clone and used to prepare a plasmid DNA stock of the desired construct (QIAGEN maxi kit). Confirmatory sequence reactions were performed using the Big Dye terminator sequencing kit and run on an ABI 310 Prism Genetic Analyzer. Sequence analysis of both coding strands was performed using a selection of sequencing oligonucleotide primers and has yielded the following results:

30

Sequencing of pFBac-Porcine-s- $\alpha_2\delta$ -1- Δ 1064-1091-6His confirmed that the insert sequence corresponded to the nucleic acid encoding the polypeptide of SEQ ID No 37, except for the deletion of two bases from the 5' end of the 5' PCR primer (JB189). The

loss of these two bases did not have any impact on the 5' end of the gene as the KOZAK translation start-site consensus sequence (GCCACC) starts immediately after this deletion.

5 **Example 3**

Protocol for establishing baculovirus banks for the expression of the $\alpha_2\delta-1$ deletion mutant of SEQ ID N°9

Essentially, the protocol used to generate the baculovirus banks is that outlined in the Life Technologies Bac-to BacTM baculovirus expression systems manual.

10

a) Transposition of DH10Bac *E. Coli* cells

One ng (5 μ l) of the recombinant pFastBac-1 construct containing the nucleotide sequence encoding the porcine $\alpha_2\delta-1$ deletion mutant of SEQ ID No 37 was added to 100 μ l of DH10Bac cells thawed on ice. The cells were then mixed gently by tapping the 15 tube then incubated on ice for 30 minutes before heat shock treatment by incubation in a 42°C water bath for 45 seconds. The mixture was then chilled on ice for 2 minutes before the addition of 900 μ l of S.O.C. medium. The mixture was then placed in a shaking incubator (200rpm) at 37°C for 4hours. The cells were then serially diluted (10 fold dilutions from 10⁻¹ to 10⁻³) and 10 μ l of each dilution plated on LB agar plates containing 20 50 μ g/ml kanamycin, 7 μ g/ml gentamicin, 10 μ g/ml tetracycline, 100 μ g/ml Bluo-gal and 40 μ g/ml IPTG. The plates were incubated at 37°C for between 1 and 3 days until discrete colonies of blue and white colour were discernible.

b) Isolation of recombinant DNA

25 White colonies (containing the recombinant bacmid) were picked and grown for 24 hours (to stationary phase) at 37°C with shaking (200 rpm) in 2ml of LB containing 50 μ g/ml kanamycin, 7 μ g/ml gentamicin and 10 μ g/ml tetracycline. 1.5ml of culture was then transferred to a microfuge tube and centrifuged at 14,000xg for 1minute. The supernatant was removed and the cells resuspended gently in 0.3ml of 15mM Tris-HCl (pH8.0), 30 10mM EDTA, 100 μ g/ml RNase A. 0.3ml of 0.2N NaOH, 1% SDS was then added and the mixture mixed gently before incubation at 22°C for 5 minutes. Then 0.3ml of 3M potassium acetate (pH5.5) was added and the sample placed on ice for 10 minutes. After

centrifugation at 14,000 x g for 10 minutes the supernatant was transferred to a tube containing 0.8ml of isopropanol, mixed then placed on ice for 10 minutes before centrifugation at 14,000 x g for 10 minutes. The supernatant was then discarded and the pellet rinsed with 0.5ml of 70% ethanol before centrifugation at 14,000 x g for 5 minutes.

5 This 70% ethanol rinse was then repeated before removing all of the supernatant and air drying the pellet for 10 minutes at room temperature. The pellet was finally resuspended in 40 μ l of TE.

c) Transfection of sf9 cells with the recombinant bacmid DNA

10 A 6-well tissue culture plate was seeded with 0.9×10^6 sf9 cells (cells at log phase having grown from a culture passaged at 0.3×10^6 cells/ml) per 35mm well in 2ml of Sf-900 II SFM media containing 50units/ml penicillin and 50 μ g/ml streptomycin. Cells were left to attach at 27°C for 1 hour. Bacmid DNA prepared as described above (5 μ l) was added to 200 μ l of Sf-900 II SFM media containing 6 μ l of CELLFECTIN and mixed before

15 incubation at room temperature for 45 minutes. The cells were washed once with 2ml of Sf-900 II SFM media without antibiotics then 0.8ml of Sf-900 II SFM media was added to each tube containing the lipid-DNA complex. The wash buffer was removed from the cells and the 1ml of diluted lipid-DNA complex overlaid on the cells. The cells were incubated for 5 hours at 27°C after which time the transfection mixture was removed and

20 2ml of Sf-900 II SFM media containing 50units/ml penicillin and 50 μ g/ml streptomycin was added. The cells were then incubated for 72 hours.

After incubation for 72 hours the media was removed from the cells and centrifuged at 500xg for 5 minutes. The supernatant was then transferred to a fresh tube, this was

25 labelled as the P0 bank and stored at 4°C in the dark. The P1 bank was prepared by passaging sf9 cells at approx 5×10^6 cells/ml to 2×10^6 cells/ml (100ml in a 250ml Erlenmeyer flask) and adding 0.5ml of the P0 bank harvested above. The cells were then incubated shaking (200rpm) at 27°C for 4 days. Under sterile conditions the culture was centrifuged at 500xg for 10 minutes and the supernatant 0.2 μ M filtered (P1 bank). The

30 P2 bank was prepared by adding 2ml of P1 bank per 400ml culture (in 1L Erlenmeyer flasks) passaged as above to 2×10^6 cells/ml. The culture was incubated as before for 4

days and the supernatant harvested and filtered as described for the P1 bank. The supernatant was first pooled then aliquoted (10ml) and stored at 4°C.

Example 4

5 **Protein expression**

To sf9 cells passaged from $\sim 5 \times 10^6$ cells/ml to 2×10^6 cells/ml in Sf-900 II SFM media was added 0.1ml virus per 100 ml of cells of the appropriate viral bank (400ml volumes in 1L Erlenmeyer flasks). The cells were then cultured for 4-5 days at 27°C with 110 rpm shaking. Expression of the protein was confirmed by SDS-PAGE and Western blotting 10 using an anti penta-His monoclonal antibody (Qiagen) and was detected in the culture supernatant and cell lysate.

Example 5

Purification of $\alpha_2\delta$ -1deletion mutant of SEQ ID N°37

15 The- $\alpha_2\delta$ -1deletion mutant of SEQ ID N°37 was purified from the cell lysate following the purification strategy outlined below:

The culture was centrifuged at 6,000xg for 10 minutes and the supernatant removed. The weight of the cell pellet was determined before re-suspension in 20mM Tris pH8.0, 20 100mM KCl, 1% P40-Nonidet (100ml per 20g of wet cells). A protease inhibitor cocktail (Sigma, Cat# P8849), 1ml/L, was added to the mixture. The solution was then stirred for 10 minutes before centrifugation for 1hour at 30,000xg and 4°C. The supernatant was concentrated (30kDa cut off) to approx. ~300ml then centrifuged for 1hour at 100,000xg. 25 Supernatant containing the soluble proteins was diluted 1:3 in 10mM Tris-HCl pH8.0 (equilibration buffer) and loaded onto a pre-equilibrated Q-Sepharose column (2.5cm i.d. x 30cm h.) at a flow rate of 900ml/h. After washing with equilibration buffer until a stable $A_{280\text{nm}}$ baseline had been achieved, protein was eluted with 20mM Tris-HCl pH8.0, 0.5M KCl, 10mM Imidazole.

30

The eluate was then loaded onto a Ni-NTA (Qiagen) column (2.5cm i.d. x 6cm h.) pre-equilibrated in 20mM Tris pH8.0, 0.5M KCl, 10mM Imidazole at a flow rate of 2

ml/min. The column was washed successively with buffer A (20mM Tris pH8.0, 0.5M KCl, 20mM Imidazole), buffer B (100mM Tris-HCl pH8.0, 1M KCl), and buffer A again. Elution was performed with buffer C (20mM Tris-HCl pH8.0, 100mM KCl, 0.5M Imidazole). The Ni-NTA eluate (~50ml) was concentrated (30kDa cut-off) to ~2ml and 5 applied at 1ml/min and in 0.2ml aliquots, to an FPLC Superdex-200 column equilibrated in 10mM HEPES, pH7.4, 150mM NaCl. Fractions containing the polypeptide of SEQ ID No 37 were pooled. As shown in Figure 1, the protein elution profile and associated [³H]gabapentin binding activity is presented together with a silver-stained SDS-PAGE gel (post Ni NTA load of Superdex-200) demonstrating the co-elution of the ~130kDa 10 band ($\alpha_2\delta$) with the [³H]gabapentin binding activity and $A_{280\text{nm}}$ profile.

Example 6

SPA assay of [³H]gabapentin binding to soluble porcine $\alpha_2\delta$ -1b-6His

The assay was carried out at 21°C. Assay components were added in the following order 15 (all reagents were diluted in 10mM HEPES (pH 7.4 at 21°C) to 96-well Optiplates)

25 μ l imidazole at various concentrations (diluted from a 1M stock pH8.0, see assay details)
50 μ l 10mM HEPES pH 7.4
25 μ l (50mg) SPA beads (Amersham)
20 100 μ l s- $\alpha_2\delta$ -1b-6His of SEQ ID No 9 (2 μ l protein diluted to 100 μ l) obtained from example 5

25 μ l radioligand ([³H]gabapentin)

Immediately after adding radioligand, the optiplates were loaded in the Packard Top Count scintillation counter to follow the binding time course. Imidazole was first used in 25 the assay to optimize the specific interaction of the protein's 6His tag with the SPA bead. Imidazole itself (up to 100mM) in the filtration assay has no effect on [³H]gabapentin binding (n=1).

As shown in figure 2, specific binding of [³H]gabapentin to the s- $\alpha_2\delta$ -1b-6His was 30 enhanced by imidazole. Of the concentrations, tested the optimal was 50mM. Equilibration was reached after ~2hours.

Example 7**Ni Flashplate assay of [³H]gabapentin binding to soluble porcine $\alpha_2\delta$ -1b-6His (SEQ****ID No 37)**

Assays were carried out at 21°C in a final volume of 250µl in 96-well NEN Ni chelate 5 flash plates. Assay components were added in the following order (all reagents were diluted in 10mM HEPES (pH 7.4 at 21°C)):

25µl 10mM HEPES pH7.4
25µl imidazole at various concentrations (diluted from a 1M stock pH8.0, see assay details)
10 75µl 10mM HEPES pH 7.4
100µl s- $\alpha_2\delta$ -1b-6His (2µl protein diluted to 100µl) obtained from example 5
25µl radioligand ([³H]gabapentin)

15 Immediately after adding the radioligand, flash plates were loaded in the Packard Top Count scintillation counter to follow the binding time course. The '[³H] flash plate' programme (cpm) was used to monitor activity. Imidazole was first used in the assay to optimize the specific interaction of the protein's 6His tag with the Ni flashplate. Imidazole itself (up to 100mM) in the filtration assay has no effect on [³H]gabapentin 20 binding (n=1).

As shown in figure 3, the specific binding of [³H]gabapentin to the s- $\alpha_2\delta$ -1b-6His was enhanced by imidazole. Initially, from the concentrations tested, the best concentration was found to be 10mM.

25 Specific binding was determined at different volumes of s- $\alpha_2\delta$ -1b-6His, in the presence of 10mM imidazole, over a time period of 10h. Results are shown in figure 4 and equilibrium was reached at ~3h. Specific binding increased linearly with increasing amounts of protein, up to 8µl, after which the binding capacity of the Ni chelate in the assay well was probably exceeded (see figure 5). The published maximum binding 30 capacity of NEN plates is 6pmol/well. The concentration of purified s- $\alpha_2\delta$ -1b-6His is estimated at ~0.6pmol/µl, which yields 5pmol/well at 8µl.

Saturation experiments were performed with 12 duplicate data points, [³H]gabapentin concentration ranged from ~1 to 350nM. Data was analyzed using KEL-RADLIG for Windows. The results are presented in Table 2 below.

5

Table 2
Saturation studies

Flash plate (2 μ l protein used, n=2)	Filter binding K _D (nM) (4 μ l protein used, n=3)
K _D , 9.32nM K _D , 10.5nM	K _D , 12.3nM K _D , 8.91nM K _D , 10.6nM
Mean = 9.91nM	Mean = 10.60 ± 0.98nM

10 **Example 8****Ni Flashplate assay of [³H]Leucine binding to soluble porcine $\alpha_2\delta$ -1b-6His**

The procedure described in example 2 was repeated, except that [³H]gabapentin was replaced by 25 μ l (10.1 nM) of [³H]Leucine, as shown in figure 8, [³H]Leucine binds with high affinity to soluble $\alpha_2\delta$ -1b-6His. This demonstrates that it is possible to use

15 commercially available forms of [³H]Leucine in replacement of [³H]gabapentin in the assay.

Example 9**Ni Flashplate assay studying competitive binding of [³H]gabapentin, (S+)-3-isobutyl**

20 **GABA and (R)-3-isobutyl GABA to porcine $\alpha_2\delta$ -1b-6His (SEQ ID No 9)**

Assays were carried out at 21°C in a final volume of 250 μ l in 96-well NEN Ni chelate flash plates. Wells were set up for both 'total' and 'non-specific' binding. Specific binding was defined as that remaining after subtraction of the average of the 'non-specific binding' values from the average of the 'total' binding values. Assay components were added in the following order (all reagents were diluted in 10mM HEPES (pH 7.4 at 21°C)):

25 μ l 10mM HEPES pH7.4 or 25 μ l of the test compound at the appropriate concentration in HEPES

25µl 200 mM imidazole (diluted from a 1M stock pH8.0, see assay details)

Total binding 75µl 10mM HEPES pH 7.4

Non-specific binding 50µl 10mM HEPES pH 7.4 and 25µl 100µM (S+)-3-isobutyl 5 GABA

100µl s- $\alpha_2\delta$ -1b-6His (2µl protein* diluted to 100µl)

25µl radioligand ($[^3\text{H}]$ gabapentin or $[^3\text{H}]$ Leucine)

* The source of s- $\alpha_2\delta$ -1b-6His was that purified by FPLC Superdex-200 gel filtration
10 (see example 5)

Immediately after adding radioligand, flash plates were loaded in the Packard Top Count scintillation counter to follow the binding time course. Incubation time before the assay was 3 hours. The ' $[^3\text{H}]$ flash plate' programme (cpm) was used to monitor activity. Specific binding was ~98% of the 'total' value. Imidazole was used in the assay to 15 optimize the specific interaction of the protein's 6His tag with the Ni flashplate. Imidazole itself (up to 100mM) in the filtration assay has no effect on $[^3\text{H}]$ gabapentin binding (n=1).

Competition studies were compared across the flash-plate and filter binding
20 methodologies in order to validate the new assay technology with the established filter binding methodology.

GraphPad Prism software was used to process competition curve data and determine IC₅₀ and hill slope values. Twelve point competition curves with half log dilution steps of test
25 compounds were used in the experiments. Results are shown in Table 3 below where IC₅₀ values are presented, and in figure 9 where hill slopes range from -0.9 to 1.3. The $[^3\text{H}]$ Gabapentin concentration used in assay is in the range of 10-13nM

Table 3

Competition studies:

GraphPad Prism software was used to process competition curve data and determine IC₅₀ and hill slope values. Ten point competition curves with half log dilution steps of test 5 compounds were used in the experiments.

IC₅₀ values were converted to Ki values (presented in table) according to the following equation:

$$Ki = IC_{50} / (1+[L]/K_D)$$

The K_D values used were those mean values presented in table 1.

10 The [³H]Gabapentin concentration in the assay ranged from 10-13nM and was determined for each experiment for the purpose of calculating the Ki value as described above.

Hill slopes were all in the range of -0.9 to 1.3

<u>Test compound</u>	<u>Flash plate</u> (3 μ l protein used, n=2)	<u>Filter binding</u> K _D (nm) (4 μ l protein used, n=3)
Gabapentin	10.4 7.97	7.13 7.70 10.2
Mean (geometric)	9.10nM	7.84nM
(S+)-3-isobutyl GABA	10.9 7.58	6.52 6.21 8.29
Mean (geometric)	9.09nM	6.95nM
(R)-3-isobutyl GABA	157 207	78.4 64.2 107
Mean (geometric)	180nM	81.5nM

15

Example 10

Filter binding assay of [³H]gabapentin binding to the recombinant polypeptide of SEQ ID N_ 9

20

Assays were carried out at 21°C in a final volume of 250µl in 96-deep well plates. Assay components were (all reagents were diluted in 10mM HEPES (pH 7.4 at 21°C)):

25µl compound to test
200µl Polypeptide of SEQ ID No 9 (3µl protein diluted to 200µl)
5 25µl radioligand ([³H]gabapentin (65Ci/mmol)

Plates were incubated at room temperature for 1h prior to filtering on to 96-well GF/B Unifilter plates pre-soaked in 0.3% polyethylenimine. Filters were washed with 3x1ml 50mM Tris-HCl (pH 7.4 at 4°C), and dried over-night. Scintillant (Microscint O, 50µl) 10 was added and the plates counted using a Packard Top Count scintillation counter. Specific binding was ~98% of the 'total' value. In [³H]gabapentin saturation studies, the K_D (nM) obtained was about 10.62.

15 **Example 11 :**

Binding of [³H]gabapentin to the recombinant polypeptide of SEQ ID No 37 using various assay formats.

This example further illustrates the screening method of this invention, using various 20 assay formats.

a) Preparation of protein stocks

Protein was expressed as described in Example 4 except that the cells were infected at 1x10⁶ cells/ml. Additionally the cells were cultured in 20 litre Applikon fermentation 25 vessels (18L culture volume). The culture was maintained at 27°C and 60% dO₂ (100% dO₂ equates to [O₂] when media - without cells - has been saturated with air at 27°C) with single marine impeller stirring at 125rpm. The protein was expressed in either Sf-900 II SFM (LTI Inc) or ESF-921 (Expression Systems Inc.) media.

30 **b) Purification of s- $\alpha_2\delta$ -1b-6His pr tein from cell culture supernatants**

On the harvest day (day 4-7 post-infection with virus) the cell culture was centrifuged at 9,000xg for 20 minutes to remove the cellular debris, and the supernatant concentrated to

approximately 3 litres using a pellicon tangential-flow filtration system employing 30kDa cut-off regenerated-cellulose cassettes. The concentrated sample was re-centrifuged at 9,000xg for 20 minutes then diluted with 2 volumes of 10mM Tris (pH9.0). The diluted sample was then loaded at 10ml/min onto a Q-sepharose FF column 5 (5cm i.d. x 50cm h.) which was washed with 20mM Tris-HCl (pH8.0) and eluted with 20mM Tris-HCl (pH8.0), 0.5M KCl, 10mM Imidazole.

The eluate was then loaded at 10ml/min onto a Ni-superflow (Qiagen) column (2.5cm i.d. x 6cm h.) pre-equilibrated in 20mM Tris (pH8.0), 0.1M KCl, 10mM Imidazole. The 10 column was washed successively with buffer A (20mM Tris (pH8.0), 0.5M KCl, 20mM Imidazole), buffer B (20mM Tris-HCl (pH8.0), 1M KCl), and buffer A again at 10ml/min. Elution was performed with a gradient of buffer C (20mM Tris-HCl (pH8.0), 15 100mM KCl, 0.5M Imidazole) against 20mM Tris-HCl (pH8.0), 100mM KCl at 2ml/min (over 5 column volumes). Fractions from the gradient elution were assayed for [³H]gabapentin binding activity and the active fractions pooled then dialysed at 4°C four 15 times (each for 24 hours) against 10mM HEPES (pH7.3), 150mM NaCl at a ratio of 1:60 (sample:dialysate). The dialysed material was then aliquoted and frozen for use in the assays as described below.

20 **c) Preparation of protein cocktails for filter, wheat germ lectin and Ni chelate assays**
(volumes in μ l):

	cocktail	x1		x23	
		s- $\alpha_2\delta$ -1b-6His	HBS	s- $\alpha_2\delta$ -1b-6His	HBS
25	0 μ l	0	75	0	1,725
	1 μ l	1	74	23	1,702
	2 μ l	2	73	46	1,679
	4 μ l	4	71	92	1,633

30 $\alpha_2\delta$ -1 protein was sourced from the aliquots generated above.

d) Filter and Wheat Germ Lectin flashplate assays

The reagents were added in the following order to each well of either a 96-well Wheat Germ Lectin flashplate or a 96-deep well plate. Conditions were prepared in triplicate for both 'total' and 'non-specific' binding (20µl H₂O added for total binding and 20µl of 100µM (S+)-3-isobutyl GABA to define non-specific binding) for each of the four 5 volumes of protein tested.

Assay set-up per well:

	100µM (S+)-3-isobutyl GABA / H ₂ O	20µl
10	*100nM [³ H]Gabapentin	20µl
	235mM HEPES (pH7.3)	85µl
	α ₂ δ-1 (0, 1, 2 or 4µl of the x23 cocktail)	75µl

* 20µl aliquots of the [³H]gabapentin stock added to each well were counted on a liquid 15 β-scintillation counter (Beckman LS 5000TD) to determine the actual concentration of [³H]gabapentin reached in each well. For these experiments this value was calculated as 10.8nM.

The Wheat Germ lectin flashplate was then counted under continuous cycling conditions 20 on a Packard Top Count Microplate scintillation counter. The plate was counted on the '[³H]flashplate' programme with a count delay and count time of 1 minute. Data for the wheat germ lectin assay was plotted as 'specific' binding (i.e. 'total' minus 'non-specific binding'), see figure 10.

25 For the Filter assay, the binding reaction in the deep-well plate was left for 1 hour at 22°C then filtered with three 1ml washes of 4°C 50mM Tris (pH 7.4 at 4°C) onto a 96-well GF/B filter plate pre-soaked for 1 hour in 0.3% Polyethylenemine at 4°C. After leaving at 22°C to dry overnight, 45µl of Microscint-O (Packard) was added to each filter well and the plate sealed and counted in the Packard Top Count Microplate Scintillation 30 counter on the '[³H]Microscint' programme with a count delay and count time of 1 minute. The mean of the 'total' and 'non-specific' binding is presented in figure 11.

e) Nickel flashplate assay

2.35x Nickel flashplate buffer:

4.7ml 1M HEPES pH7.3

5 0.118ml 10% BSA (Sigma A7906, Fraction V (98%), Lot 57H1088) in H₂O

1.175ml 0.2M Imidazole pH7.3 (NaOH)

14.007ml H₂O

Assay set-up per well:

10

100µM (S+)-3-isobutyl GABA / H₂O 20µl

*100nM [³H]Gabapentin 20µl

2.35x Nickel Flashplate buffer 85µl

s- $\alpha_2\delta$ -1b-6His (0, 1, 2 or 4µl of the x23 cocktail) 75µl

15

* 20µl aliquots of the [³H]gabapentin stock added to each well were counted on a liquid β-scintillation counter (Beckman LS5000TD) to determine the actual concentration of [³H]gabapentin reached in the each well. For these experiments this value was calculated as 10.8nM.

20

The Nickel flashplate was then counted under continuous cycling conditions on the Packard Top Count Microplate scintillation counter. The plate was counted on the '[³H]flashplate' programme with a count delay and count time of 1 minute (Figure 12).

25 **f) Discussion**

This example demonstrates the extended stability of the assay over time (at least 100 hours), which is important if the assay format is to be used for mass-screening purposes. Indeed, such a stability enables the stacking of plates into counters (ideally with appropriate controls on each plate along with test compound wells in order to confirm 30 signal stability across individual plates).

The example also shows that it is possible to use the Wheat Germ lectin flashplate assay, as a primary assay or as a secondary screen to further refine ligands identified or selected using the Ni flashplate assay or another assay format of this invention.

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